IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Alfonso Fernández-Mayoralas Alvarez	Examiner :	Underdahl, Thane E.
Serial No.:	10/738,378	Group Unit:	1651
Filed:	December 17, 2003		

For: ENZYMATIC METHOD OF PRODUCING 4-O-β-D-GALACTOPYRANOSYL-D-XYLOSE, 4-O-β-D-GALACTOPYRANOSYL-D-XYLOSE OBTAINED USING SAID METHOD, COMPOSITIONS CONTAINING SAME AND THE USE THEREOF IN EVALUATING INTESTINAL LACTASE

SECOND DECLARATION UNDER 37 C.F.R. § 1.132

COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Alfonso Fernández-Mayoralas Alvarez, as evidenced by my signature below, declare the following:

1. I received a Ph.D. in Chemistry in 1987 from Autónoma University of Madrid (Spain). I received a Master's Degree in Chemistry in 1983 from Autónoma University of Madrid. I received a Bachelor's Degree in Chemistry in 1982 from Autónoma University of Madrid.

2. In addition to the academic appointments set forth in paragraph 1, *supra*, I was a Research Scientist at various public institutions (see Exhibit A) from 1985 - 2007.

3. My research interests include developing synthesis processes for 4-galactosyl-xylose. A detailed chronology of my academic and professional accomplishments is submitted herewith in my *Curriculum Vitae*, Exhibit A.

4. I have reviewed the above-noted United States patent application, Serial Number 10/738,378, the subject matter described therein as well as the currently pending claims in the application and Reyes *et al.*, U.S. Patent 5,994,092, Ponpipom *et al.*, U.S. Patent 4,228,274, Crumpton *et al., Biochem. J.* 70(4):729 (1958), Wong-Madden *et al.*, U.S. Patent 5,770,405, Dahmen *et al.*, U.S. Patent 4,675,392, Rao *et al., Qual. Plant.-Pl. Fds. Hum. Nutr. XXVIII* 4:293-303 (1979), Gabelsberger *et al., FEMS Letters* 109(2-3): 131 (1993), Fujimoto *et al., Glycoconjugate Journal* 15:155 (1998) and Yoshitake *et al., Eur. J. Biochem.* 101:395 (1979).

5. The claims pending in the above-referenced patent application are directed to obtaining a product useful for evaluating intestinal lactase. In the process described two parts can be distinguished, namely an enzyme reaction and a subsequent purification of the reaction mixture. Developing a method for purifying a carbohydrate mixture is neither easy nor routine for one skilled in the art, due at least in part to the characteristics of carbohydrate molecules.

6. The chemistry of carbohydrates is very complex, since different carbohydrate molecules have very similar structures. Many carbohydrates even have the same molecular formula. For example, lactose and sucrose both have 12 carbons, 22 hydrogens and 11 oxygens and the same type of functional group (the hydroxyl group).

7. Small changes in the structure of carbohydrates (for example, two carbohydrates that differ in the stereochemistry of one of their chiral centers, such as celobiose and lactose, can give rise to significant differences both in chemical reactivity and in behavior in the purification processes. Raymond Lemieux opined the following: "The only generalization that exists in the chemistry of carbohydrates is that there is no generalization."

8. Applicants submit herewith two additional journal articles that each further describe some of the complexities and problems associated with carbohydrate chemistry, Marcaurelle *et al., Current Opinion in Chemical Biology*, 2002, 6:289-296 (Exhibit B) and Holemann *et al., Current Opinion in Biotechnology*, 2004, 15:615-622 (Exhibit C).

9. Although crystallization is in fact a common process for purifying sugars, finding the appropriate solvent is not easy. The appropriate solvent depends on the type of molecules and the range of solvents that must be tested can be very broad. The more customary solvents in sugars tend to be low molecular weight alcohols, water, ethyl acetate, hexane, and their mixtures. Applicants respectfully submit that in a crystallization process, a large number of solvents and mixtures thereof must be tested or screened before arriving at the appropriate solvent to use.

The process described in the above-referenced patent application provides products that are more than 99% pure

10. In the case of the process claimed in the above-referenced patent application, acetone allows obtaining the product desired with a >99% degree of purity. Such a purity level was not possible with more usual solvents.

11. I produced 4-O-β-D-galactopyranosyl-D-xylose enzymatically by first preparing a first reaction mixture of 2-20% by weight of D-xylose, 0.5 to 5% by weight of a β-D-galactopyranoside substrate, 75-97.5% by weight of a reaction medium that comprises buffered water at a pH between 5.0 and 9.0. I added 10 to 1,000 units of a β-D-galactosidase enzyme, per gram of β-D-galactopyranoside, to the first reaction mixture thereby obtaining a second reaction mixture. I subjected the second reaction mixture to a reaction at a temperature between a temperature higher than the freezing point of the second reaction mixture and 45° C, for 2 to 48 hours, in order to form disaccharides in the second reaction mixture. I stopped the reaction when

the disaccharides had been formed in the desired amount, by either deactivating β-D-galactosidase by freezing the second reaction mixture at a temperature between -20 ° C and -170 ° C, by deactivating β-D-galactosidase by heating the second reaction mixture to a temperature between 95 and 110 ° C, or by separating β-D-galactosidase from the second reaction mixture by ultrafiltration, thereby obtaining a third reaction mixture. I separated an aglyconic fragment of the β-D-galactopyranoside substrate used in the first step from the third reaction mixture by extraction or filtration thereby obtaining a fourth reaction mixture. I isolated fractions that contain 4-O-β-D-galactopyranosyl-D-xylose by either adding celite to the fourth reaction mixture, followed by solid-liquid extraction with a solvent and elution with a first eluent in a column or by directly adding active carbon to the fourth reaction mixture followed by filtration and elution with a second eluent. Next, I crystallized the fractions that contain 4-O-β-D-galactopyranosyl-D-xylose in a crystallization mixture of either a mixture of acetone/methanol in a ratio between 5/1 and 20/1, preferably 10/1, or a mixture of acetone/water in a ratio between 5/1 and 20/1, preferably 10/1.

12. I analyzed a sample produced by the process as outlined in paragraph 11, *supra*, and as described in the above-referenced patent application. I loaded the sample onto a GC chromatographer in order to assess the purity achieved after crystallization. GC analysis was performed on a Hewlett-Packard 5890 gas chromatographer equipped with a fused Ultra 2 Hewlett Packard column (10 m with a 0.3 mm I.d. and 0.15 μm film). A flow rate of 1 ml/min of nitrogen and a flame ionization detector were used. The temperature program was as follows: initial temperature 195°C for 2 minutes followed by an increase of 5°C/minute to 250°C for the remainder of the run. Prior to injection, samples were silylated using a mixture of trimethylsilyl imidazol and pyridine (1:1, v/v) at 60°C for 30 minutes. One microliter of the silylated sample was analyzed by GC.

13. Exhibit D is a gas chromatogram of the 4-galactosyl-xylose obtained by the process described in the above-referenced patent application and in paragraph 11, *supra*. The

sample was analyzed as described in paragraph 12, *supra*. Peaks at retention times of 18.70 and 18.92 min correspond to alpha and beta anomers of 4-galactosyl-xylose, respectively. By simply summing the % areas of each peak (92.566 + 6.540 = 99.106%), a purity of over 99% is achieved.

14. Wong-Madden *et al.*, U.S. Patent 5,770,405 do not use the solvent mixture in a chromatography on active carbon. Wong-Madden *et al.* teach using isopropanol/ethanol/water, but it is to develop a chromatography on silica gel. (*See*, Column 33, line 25).

15. Active carbon is customarily used to eliminate hydrophobic impurities, but it is not normally used in organic synthesis, for separating monosaccharide and disaccharide mixtures, such as is the case in the above-referenced patent application. The normal course to separate these mixtures is to employ chromatography on a silica gel, on sepharose or others (*See,* Wong-Madden et al., Column 11, line 19). The above-referenced patent application describes purifying a mono- and disaccharide mixture using active carbon, which offers the advantage, compared with usual adsorbents (e.g., silica gel or sepharose) of being cheaper. In H. Rotzche, *Journal of Chromatography Library* 1991, 48:104-107 (Exhibit E), either structural and geometrical differences between each kind of adsorption matrixes, active carbon in comparison with other column fillings as sepharose, silica gel, etc. are discussed in detail.

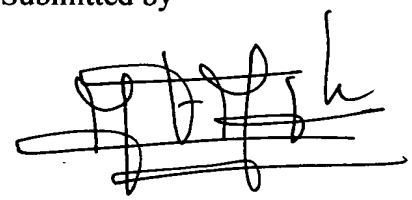
16. The above-referenced patent application describes an isopropanol/water mixture as eluent, as opposed to the more common alcohol/water mixtures such as methanol/water or ethanol/water. The methods described in the above-referenced patent application thereby provide the advantage of allowing for less elution volume, a significant advantage for industrial production (Exhibit F). Moreover, ethanol and methanol are more toxic than an isopropanol/water mixture.

17. Rao *et al.* teach extraction with Soxhlet to extract fats from a specimen of plant

origin. Rao *et al.* do not describe using Soxhlet for selectively extracting monosaccharides from a mixture of sugars.

18. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, or any patent issuing thereon.

Submitted by



Alfonso Fernández-Mayoralas Alvarez

Date Signed: September 19, 2007